

## Supplementary Materials for

### PD-1 suppresses the maintenance of cell couples between cytotoxic T cells and target tumor cells within the tumor

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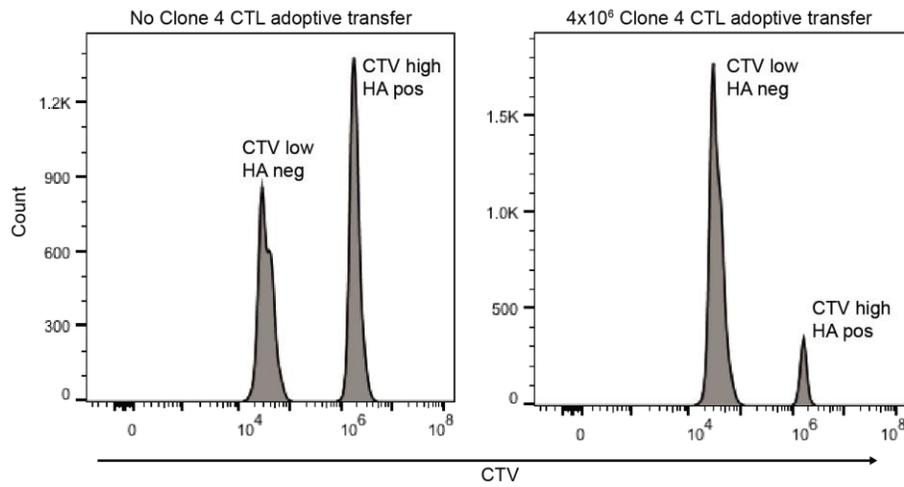
#### Other Supplementary Material for this manuscript includes the following:

(available at [stke.sciencemag.org/cgi/content/full/13/649/eaau4518/DC1](http://stke.sciencemag.org/cgi/content/full/13/649/eaau4518/DC1))

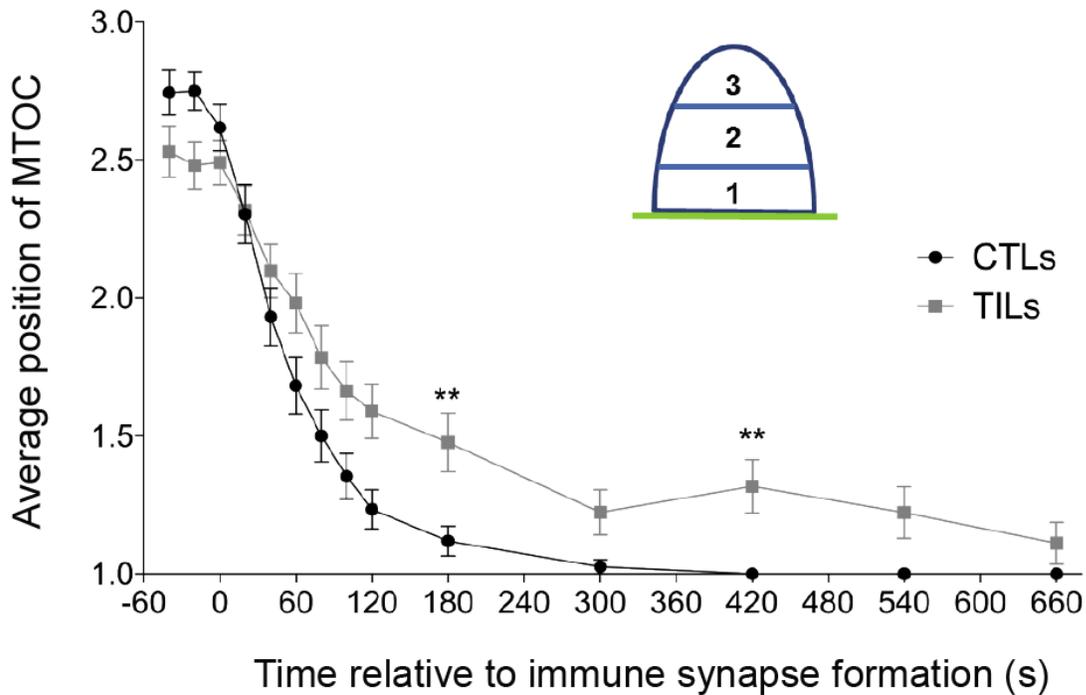
- Movie S1 (.mp4 format). Representative interactions of a tubulin-GFP-expressing clone 4 CTL and TIL with Renca<sup>+HA</sup> target cells.
- Movie S2 (.mp4 format). Representative interactions of an F-tractin-GFP-expressing clone 4 CTL, TIL, and Jasplakinolide-treated CTL with Renca<sup>+HA</sup> target cells.
- Movie S3 (.mp4 format). Representative interactions of a cofilin-GFP-expressing clone 4 CTL and TIL with Renca<sup>+HA</sup> target cells.
- Movie S4 (.mp4 format). Representative interaction of a coronin 1A-GFP-expressing clone 4 CTL with a Renca<sup>+HA</sup> target cell.
- Movie S5 (.mp4 format). Representative interactions of an Arp3-GFP-expressing clone 4 CTL and TIL with Renca<sup>+HA</sup> target cells.

Movie S6 (.mp4 format). Representative interaction of a chronophin-GFP-expressing clone 4 CTL with a Renca<sup>+HA</sup> target cell.

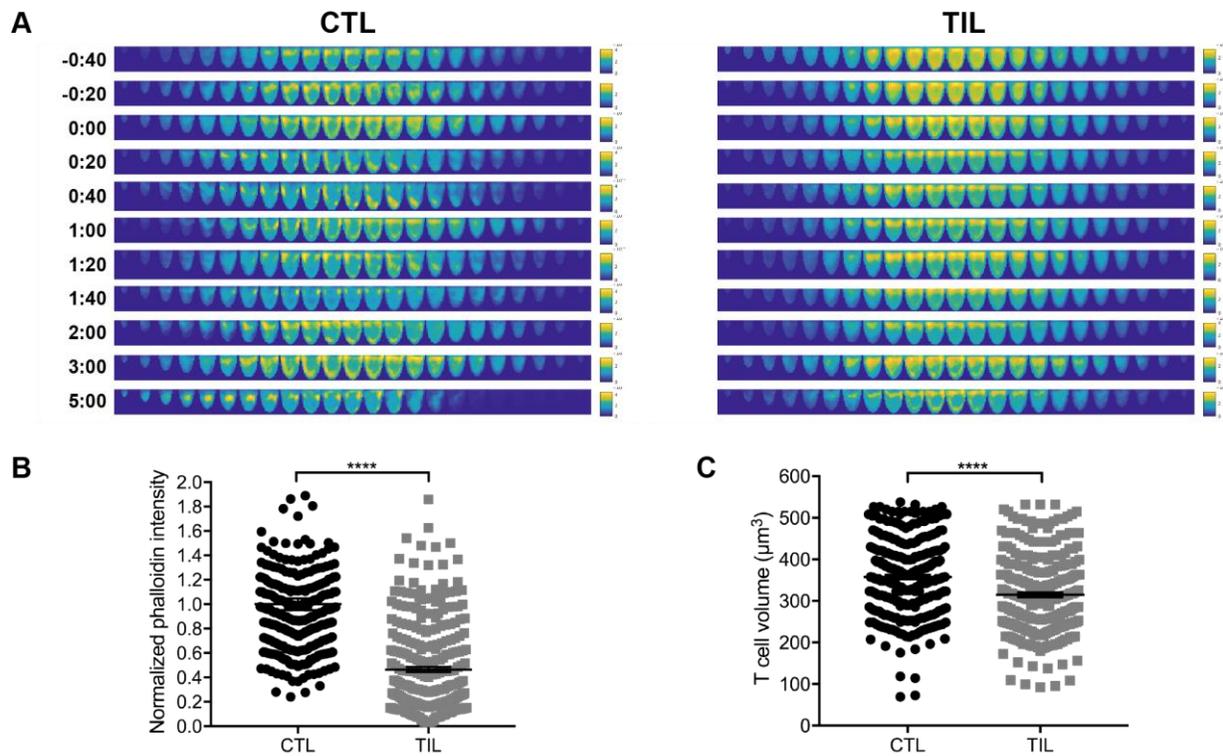
Movie S7 (.mp4 format). Representative interactions of a Fura-2-loaded clone 4 CTL and TIL with a Renca<sup>+HA</sup> target cell.



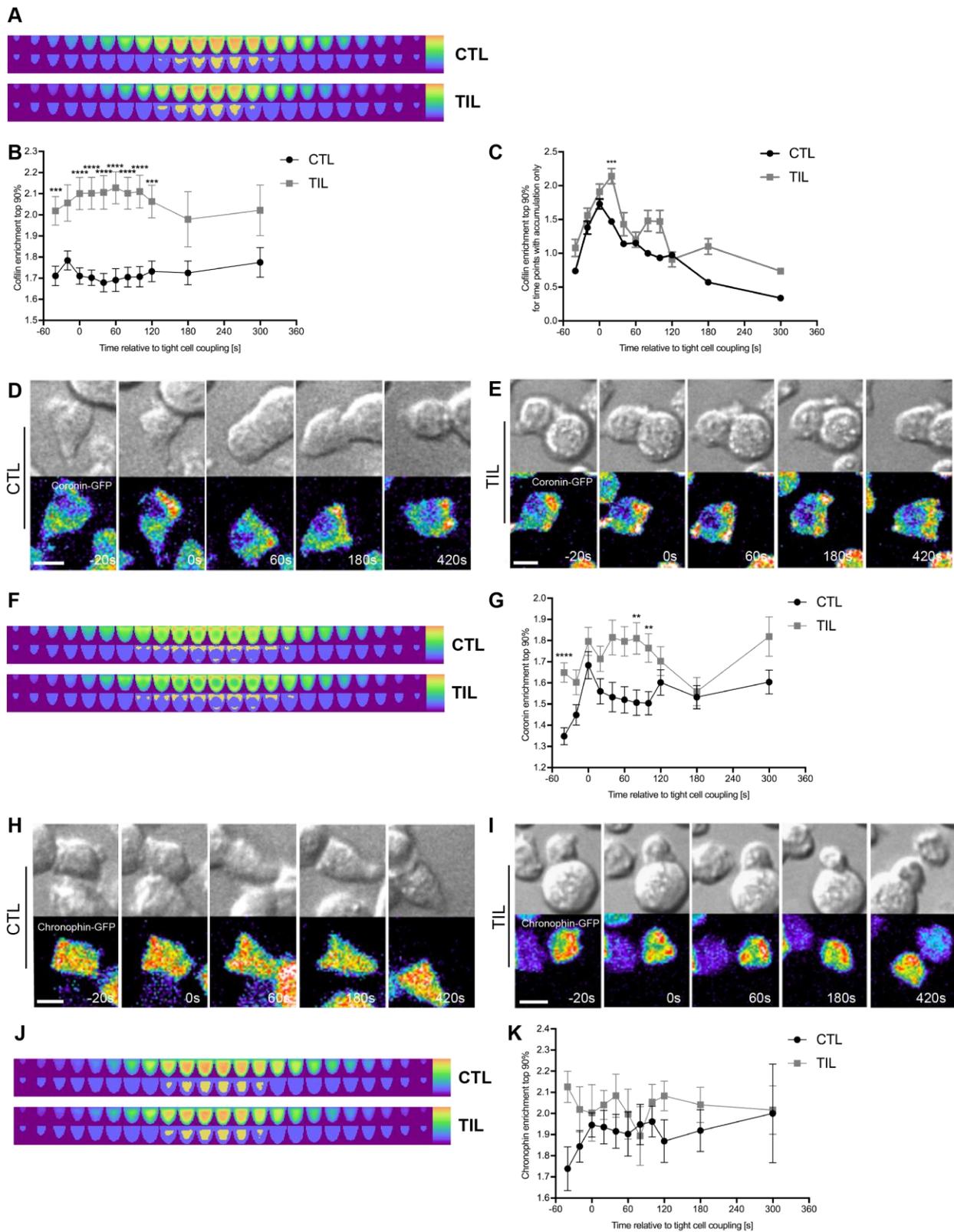
**Fig. S1. Clone 4 CTLs efficiently kill K<sup>d</sup>HA-pulsed splenocytes in vivo.** Flow cytometry analysis of the in vivo killing of HA-pulsed (CTV-high) and unpulsed (CTV-low) syngeneic splenocytes in BALB/c mice that did (right) or did not (left) receive Clone 4 CTLs 48 hours before being injected with labeled splenocytes. Histograms of live CTV<sup>+</sup> cells are representative of two independent experiments.



**Fig. S2. Diminished MTOC polarization of clone 4 TILs toward the T cell–target cell interface.** Fluorescence microscopy analysis of interactions between tubulin-GFP-expressing Clone 4 CTLs or TILs and HA peptide-pulsed Renca<sup>+HA</sup> cells over time. Position of MTOC relative to cell interface data are means  $\pm$  SEM from at least three independent experiments (shown in Fig. 2C). T cells were divided into equal thirds (inset) and position was classed as part of regions 1, 2, or 3 or as 1.5 or 2.5 if the MTOC fell on a division line. **\*\*** $P < 0.0036$  (to account for Bonferroni correction) by Mann-Whitney u-test.

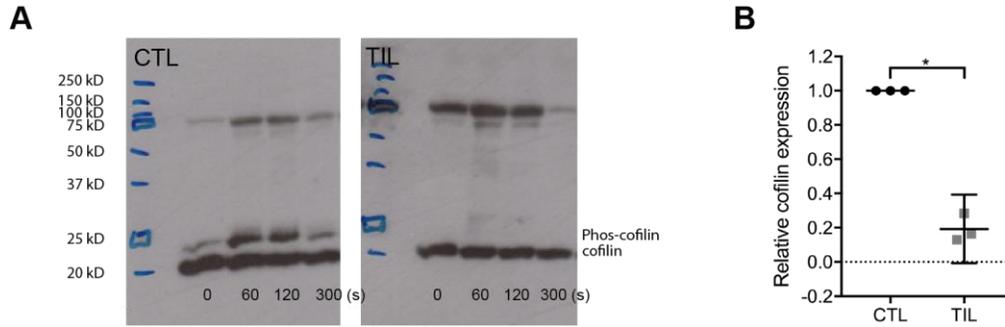


**Fig. S3. F-actin distribution is impaired in TILs.** (A) Computational analysis of F-actin-GFP reporter fluorescence distributions in CTLs (left) and TILs (right) is displayed in horizontal slices perpendicular to the synapse averaged over all cells at the indicated times from the imaging data shown in Fig. 3, A and B. (B and C) Fluorescence microscopy analysis of the amount of F-actin in CTLs and TILs. Quantified phalloidin staining intensity (B) and T cell volume (C) are means  $\pm$  SEM from two independent experiments. \*\*\*\* $P < 0.0001$  by Student's *t* test.

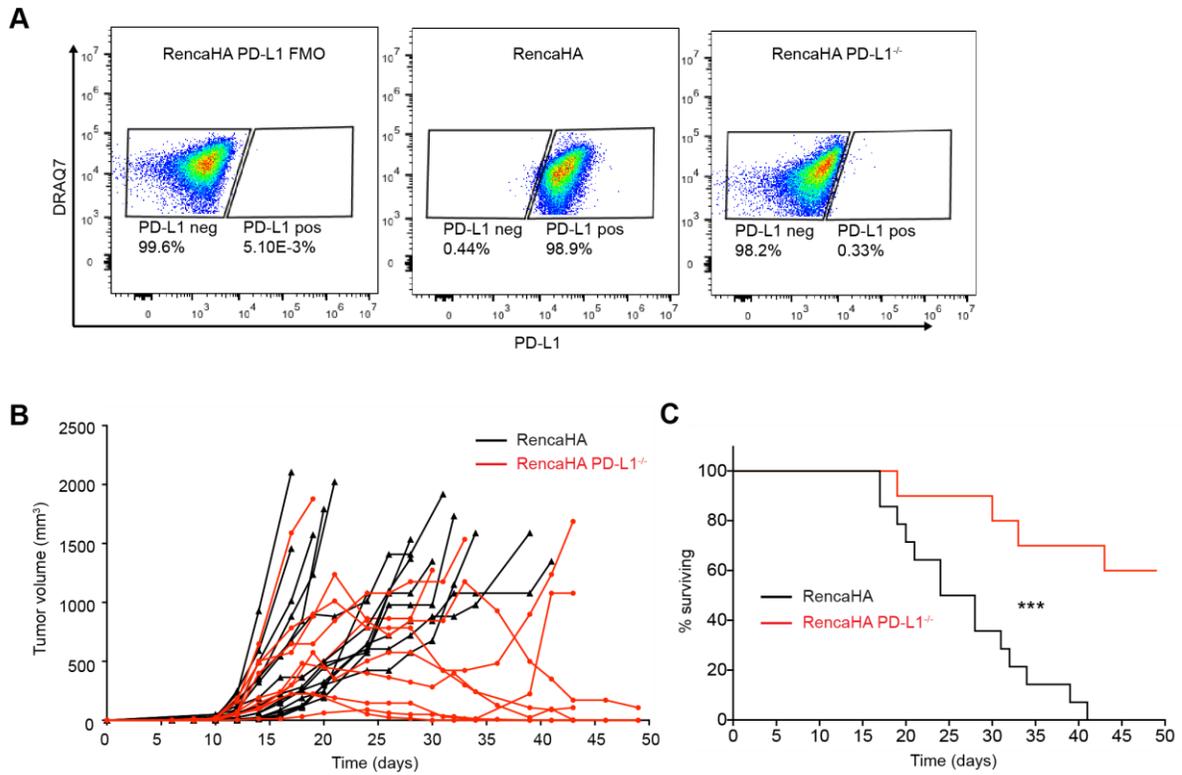


**Fig. S4. Greater enrichment of cofilin, coronin 1A, and chronophin in TILs than in CTLs.** (A to C) Analysis of Cofilin-GFP distribution in transduced Clone 4 CTLs and TILs interacting with HA peptide-pulsed Renca<sup>+HA</sup> cells over time. Images (A) were computationally generated from all of the fluorescence

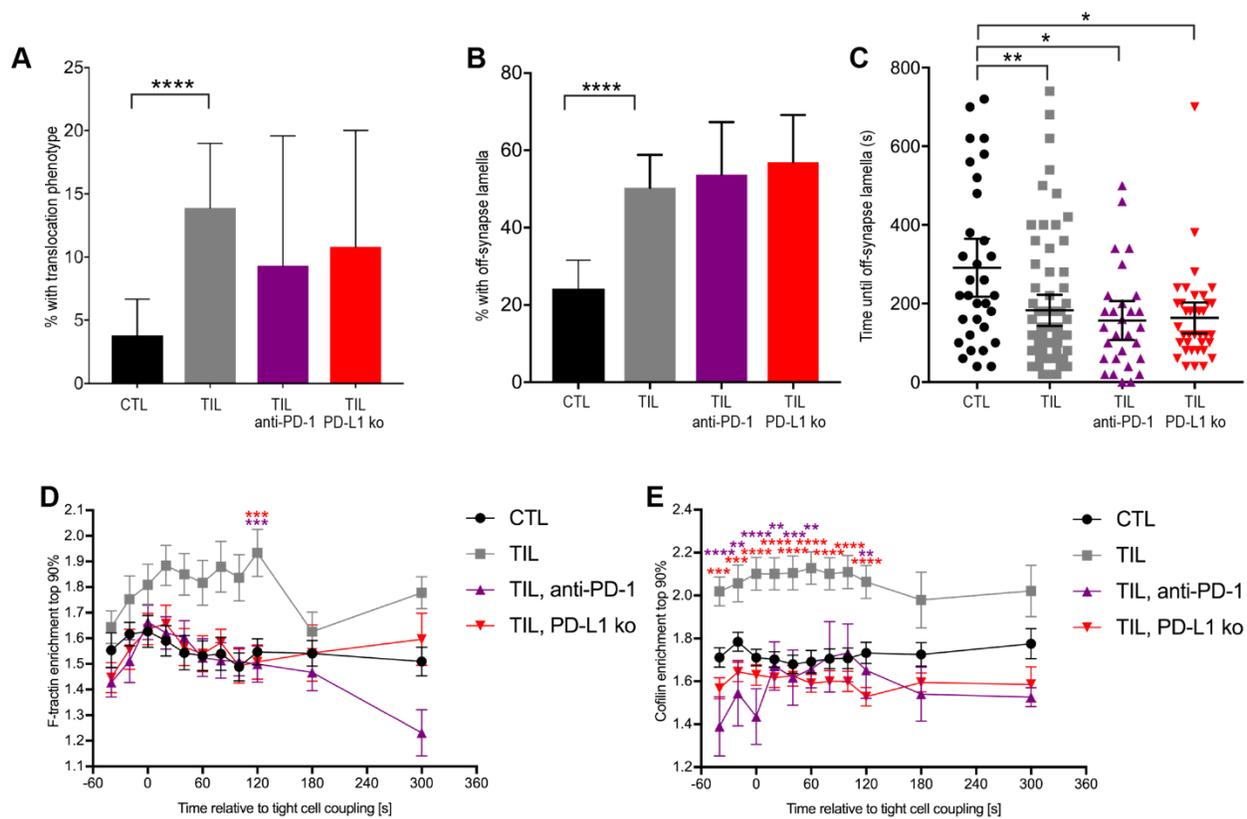
microscopy data in Fig. 4, C and D, and are displayed with the location of the 10% of the cell volume with the most intense cofilin accumulation highlighted in yellow. (B and C) Relative enrichment of intracellular cofilin-GFP in the highlighted volumes (B) and accumulation in the interface cylinder (Fig. 4C) as adjusted for cells with accumulation above background (C) are means  $\pm$  SEM from at least three experiments. (D to G) Microscopy analysis of coronin1A-GFP-expressing Clone 4 CTLs and TILs interacting with HA peptide-pulsed Renc<sup>+HA</sup> cells over time. (D and E) DIC (top) and fluorescence (bottom) microscopy images are representative of at least two experiments. (F) Computational analysis of GFP fluorescence distribution is displayed in horizontal slices perpendicular to the synapse averaged over all cells and time points with the location of the 10% of the cell volume with the most intense coronin1A-GFP accumulation highlighted in yellow. (G) Quantification of coronin1A enrichment in CTLs and TILs in the highlighted volumes are means  $\pm$  SEM of 52 CTLs and 60 TILs from all experiments. (H to K) Microscopy analysis of chronophin-GFP-expressing Clone 4 CTLs and TILs interacting with HA peptide-pulsed Renc<sup>+HA</sup> cells over time. (H and I) DIC (top) and fluorescence (bottom) microscopy images are representative of at least two experiments. (J) Computational analysis of GFP fluorescence distribution is displayed in horizontal slices perpendicular to the synapse averaged over all cells and time points with the location of the 10% of the cell volume with the most intense chronophin-GFP accumulation highlighted in yellow. (K) Quantification of chronophin enrichment in CTLs and TILs in the highlighted volumes are means  $\pm$  SEM of 58 CTLs and 41 TILs from all experiments. Scale bars, 5  $\mu$ m. \*\* $P$  < 0.0045 (to account for Bonferroni correction), \*\*\* $P$  < 0.001, and \*\*\*\* $P$  < 0.0001 by Student's  $t$  test.



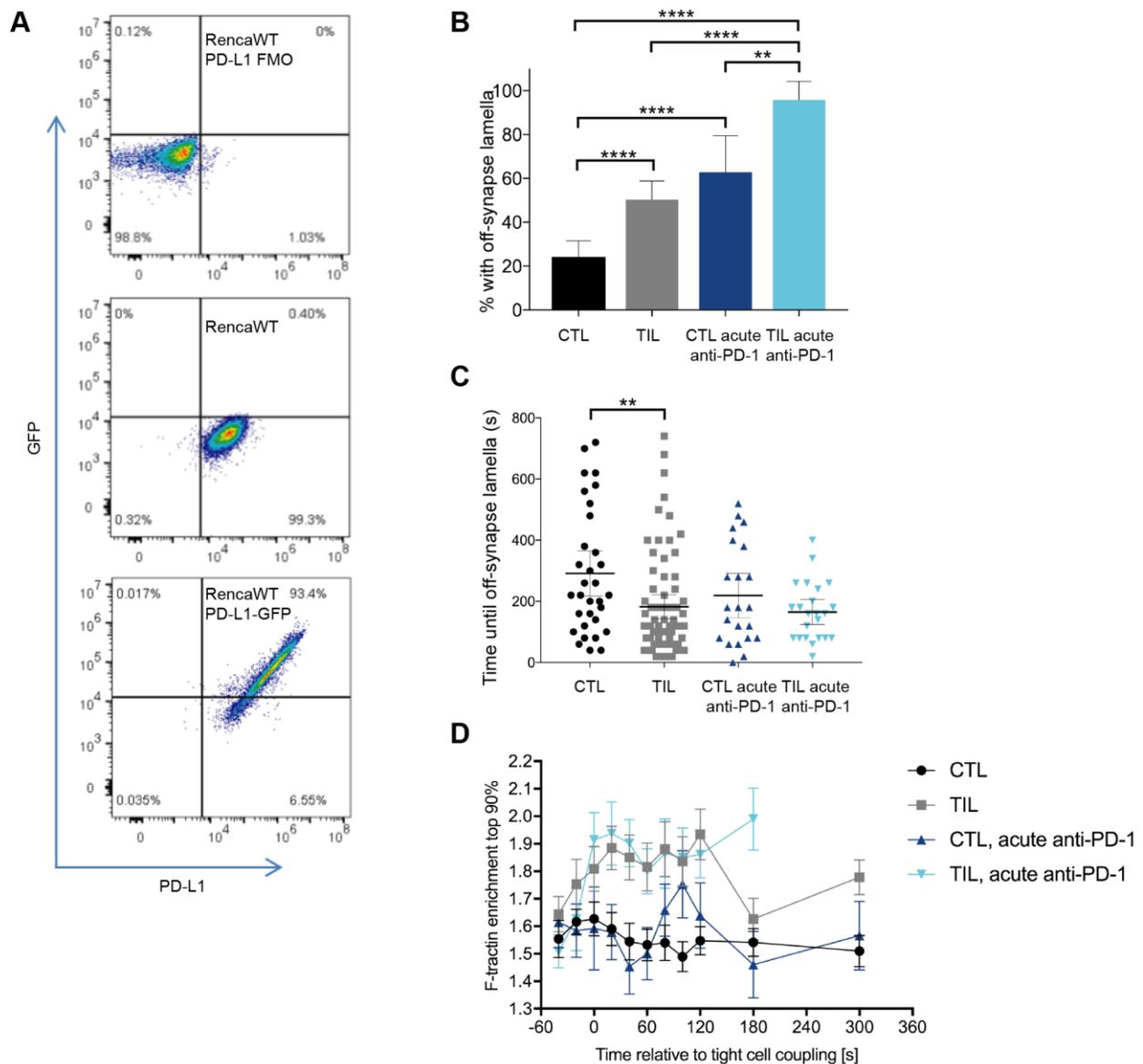
**Fig. S5. Increased cofilin phosphorylation but reduced cofilin abundance in TILs.** (A) Clone 4 CTLs and TILs were activated with antibodies against CD3 for the indicated times. The cells were then lysed and analyzed by Phos-tag Western blotting with antibodies against cofilin. Blots are representative of three experiments in Fig. 4H. (B) Quantification of the total amounts of cofilin as determined by Western blotting analysis of lysates of HA-specific CTLs and TILs. Data are means  $\pm$  95% confidence interval as normalized to the abundance of cofilin in CTLs from three independent experiments. \* $P < 0.05$  by Student's  $t$  test with Welch's correction for unequal variance.



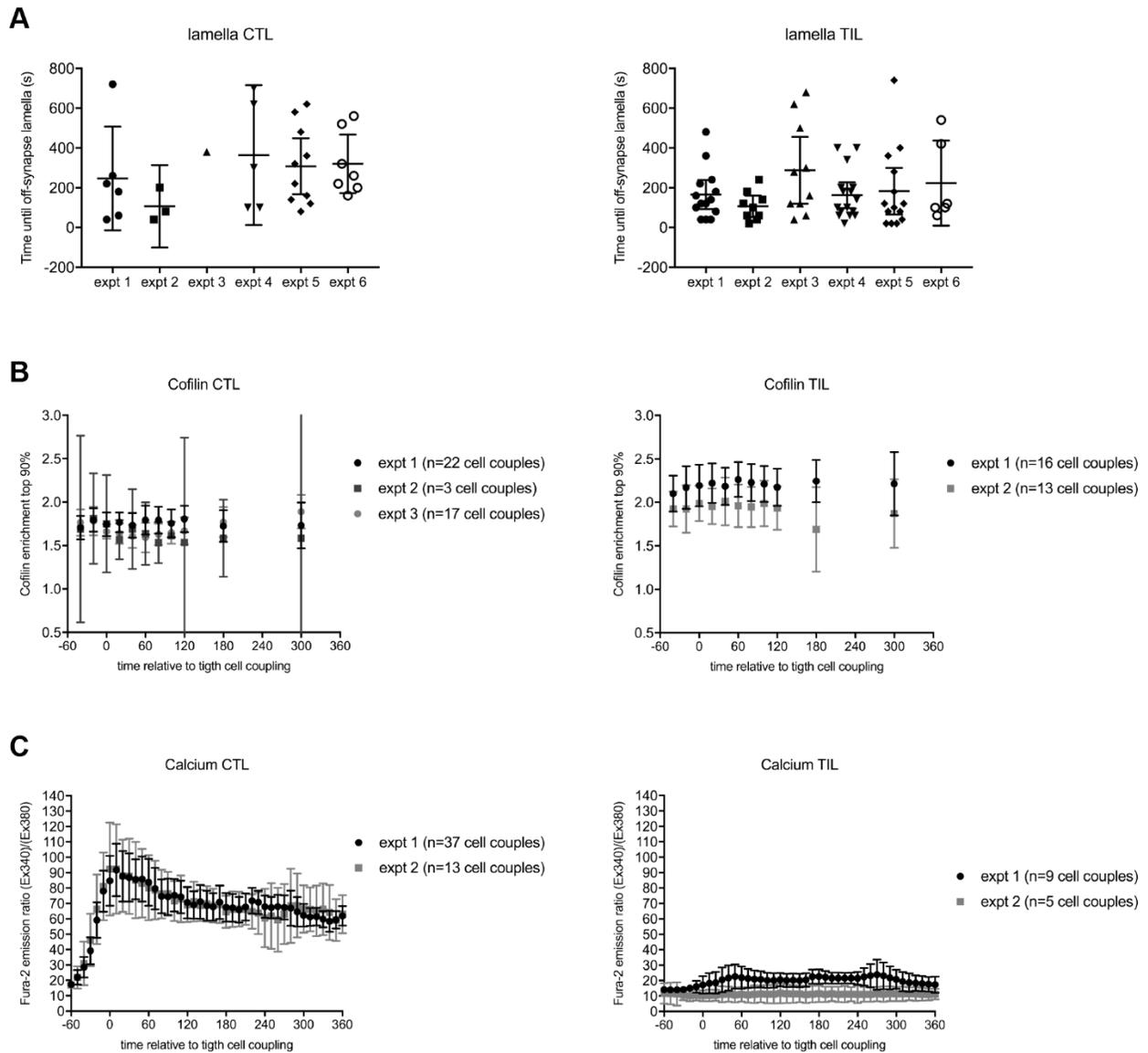
**Fig. S6. Characterization of the RencaHA PD-L1<sup>-/-</sup> cell line and corresponding tumors.** (A) Flow cytometric analysis of the amount of surface PD-L1 on live RencaHA and RencaHA-PD-L1<sup>-/-</sup> cells. Dot plots are representative of two independent experiments. (B and C) Analysis of tumor growth (B) and survival (C) after subcutaneous inoculation of mice with RencaHA or RencaHA PD-L1<sup>-/-</sup> tumors, as indicated. Data from 14 RencaHA and 10 RencaHA PD-L1<sup>-/-</sup> tumor-bearing mice were pooled from two independent experiments. \*\*\* $P < 0.001$  by Mantel-Cox test.



**Fig. S7. Loss of PD-1 engagement in vivo rescues cytoskeletal polarization.** (A to C) Microscopy analysis of HA-specific CTLs and TILs from the indicated mice interacting with Renca<sup>+HA</sup> cells. The percentage of cells that translocated more than one immune synapse diameter (A), the percentage of cells with off-synapse lamellae (B), and time at which the first off-synapse lamella was observed (C) are means  $\pm$  95% confidence interval of 138 CTLs, 137 TILs (from Fig. 2, F to H), 35 TILs from mice treated with antibody against PD-1, and 28 TILs from RencaHA PD-L1<sup>-/-</sup> tumors from at least two independent experiments. (D) Analysis of the relative enrichment of F-tractin-GFP in the 10% of the cell volume with the most intense F-tractin-GFP accumulation in F-tractin-GFP-expressing Clone 4 CTLs and TILs from the indicated mice after interacting with HA peptide-pulsed Renca<sup>+HA</sup> cells. Data are means  $\pm$  SEM from the computational analysis of the microscopy data in Fig. 3E and Fig. 8A. (E) Analysis of the relative enrichment of cofilin-GFP in the 10% of the cell volume with the most intense cofilin-GFP accumulation in cofilin-GFP-expressing Clone 4 CTLs and TILs from the indicated mice after interacting with HA peptide-pulsed Renca<sup>+HA</sup> cells. Data are means  $\pm$  SEM from the computational analysis of the microscopy data in fig. S4B and Fig. 8B. \* $P$  < 0.05, \*\* $P$  < 0.01 (C); \*\* $P$  < 0.0045 (D and E, to account for Bonferroni correction); \*\*\* $P$  < 0.001, \*\*\*\* $P$  < 0.0001 by proportion's z-test (A and B), Kruskal-Wallis test with comparison to CTLs (C), or one-way ANOVA (D and E).



**Fig. S8. Acute in vitro blockade of PD-1 does not restore cytoskeletal polarization.** (A) Flow cytometric analysis of the percentages of RencaWT and RencaWT-PD-L1-GFP cells that expressed GFP and PD-L1. Dot plots are representative of two independent experiments. (B and C) Microscopy analysis of HA-specific CTLs and TILs interacting with HA peptide-pulsed Renca<sup>+HA</sup> cells in the presence of antibody against PD-1, as indicated. The percentage of cells with off-synapse lamellae (B) and the time at which the first off-synapse lamella was observed (C) are means  $\pm$  95% confidence interval from the computational analysis of the microscopy data in Fig. 2, F and G, and Fig. 9B. (D) Analysis of the relative enrichment of F-tractin-GFP in the 10% of the cell volume with the most intense F-tractin-GFP accumulation in F-tractin-GFP-expressing Clone 4 CTLs and TILs in the presence of antibody against PD-1 after interaction with HA peptide-pulsed Renca<sup>+HA</sup> cells. Data are means  $\pm$  SEM from the computational analysis of the microscopy data in Fig. 3E and Fig. 9C. \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$  by proportion's z-test (B) or Kruskal-Wallis test with comparison to CTL (C).



**Fig. S9. Data pooling.** (A to C) Data from the indicated number of independent experimental repeats (“expt”) with the indicated number of cell couples analyzed (“n”) are given for three representative data sets comparing CTLs (left) with TILs (right) for (A) time until off-interface lamella (Fig. 2G), (B) cofilin enrichment at the cellular interface (fig. S4B), and (C) increase in the cytoplasmic  $\text{Ca}^{2+}$  concentration (Fig. 5D). Shown are individual measurements with mean  $\pm$  95% confidence interval (A) or means  $\pm$  95% confidence interval (B and C).

**Movie S1. Representative interactions of a tubulin-GFP-expressing clone 4 CTL and TIL with Renca<sup>+HA</sup> target cells.** DIC images are shown on the top, with matching top-down, maximum projections of 3D sensor fluorescence data on the bottom. The sensor fluorescence intensity is displayed in a rainbow-like, false-color scale (increasing from blue to red). Intervals (20 s) in video acquisition are played back as two frames/s. The Clone 4 CTL and TIL in Movie S1 were transduced to express tubulin-GFP. The movie starts with the CTL interaction, with cell coupling occurring on top of the image in frame 8. The TIL interaction follows with cell coupling occurring in frame 6 of the TIL section.

**Movie S2. Representative interactions of an F-tractin-GFP-expressing clone 4 CTL, TIL, and Jasplakinolide-treated CTL with Renca<sup>+HA</sup> target cells.** The Clone 4 CTLs and TIL in Movie S2 were transduced to express F-tractin-GFP. The movie starts with the CTL interaction, with cell coupling occurring in frame 4. The TIL interaction follows, with cell coupling occurring in frame 8 of the TIL section. The CTL interaction in the presence of 40 nM Jasplakinolide follows, with cell coupling occurring in frame 9.

**Movie S3. Representative interactions of a cofilin-GFP-expressing clone 4 CTL and TIL with Renca<sup>+HA</sup> target cells.** The Clone 4 CTL and TIL in Movie S3 were transduced to express cofilin-GFP. The movie starts with the CTL interaction, with cell coupling occurring on top of the image in frame 4. The TIL interaction follows, with cell coupling occurring in frame 3 of the TIL section.

**Movie S4. Representative interaction of a coronin 1A-GFP-expressing clone 4 CTL with a Renca<sup>+HA</sup> target cell.** The Clone 4 CTL in Movie S4 was transduced to express coronin 1A-GFP. Cell coupling occurs in frame 3.

**Movie S5. Representative interactions of an Arp3-GFP-expressing clone 4 CTL and TIL with Renca<sup>+HA</sup> target cells.** The Clone 4 CTL and TIL in Movie S5 were transduced to express Arp3-GFP. The movie starts with the CTL interaction, with cell coupling occurring on top of the image in frame 3. The TIL interaction follows, with cell coupling occurring in frame 4 of the TIL section.

**Movie S6. Representative interaction of a chronophin-GFP-expressing clone 4 CTL with a Renca<sup>+HA</sup> target cell.** The Clone 4 CTL in Movie S6 was transduced to express chronophin-GFP. Cell coupling occurs in frame 6.

**Movie S7. Representative interactions of a Fura-2-loaded clone 4 CTL and TIL with a Renca<sup>+HA</sup> target cell.** The Clone 4 CTL and TIL in Movie S7 were loaded with Fura-2. The ratio of Fura-2 emission upon excitation at 340 nm over that at 380 nm is given with a false color intensity scale (blue to red). Intervals (10 s) in video acquisition are played back as two frames/s. The movie starts with the CTL interaction, with cell coupling occurring on top of the image in frame 20. The TIL interaction follows, with cell coupling occurring in frame 42 of the TIL section.